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Production of antibodies against the coenzyme pyrrolequinoline quinone

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Polyclonal antibodies against pyrrolequinoline quinone have been elicited in rabbits. These antibodies react with free and protein-bound pyrrolequinoline quinone. In particular they react with native and denatured lentil seedling amine oxidase as detected by dot-blot and ELISA assays. The presence of 1 mol pyrrolequinoline quinone per mol of enzyme was determined by the last method.

Pyrrolequinoline quinone; Copper amine oxidase; Antibody; (Lentil seedling)

1. INTRODUCTION

The discovery of a new oxido-reductive coenzyme, pyrrolequinoline quinone (PQQ), has opened a new interesting area of biochemical investigation. In fact this coenzyme, first found in some dehydrogenases of prokaryotes [1], appears to be widespread in nature, being present also in yeasts, fungi, plants and animals where it may participate in different redox and non-redox reactions [2]. The complex chemical nature of PQQ suggests that at least for some organisms it may be a vitamin. The total biosynthesis of PQQ from glutamate and tyrosine has been demonstrated in bacteria [3,4].

A more thorough understanding of the distribution and metabolism of PQQ requires new sensitive analytical tools.

Up until now the determination of PQQ has been mainly performed by chromatographic or enzymatic techniques [5–8]. In the present paper we describe the production of antibodies, elicited in rabbits, which react with free and protein-bound PQQ.

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Abbreviation: PQQ, 2,7,9-tricarboxy-1H-pyrrolo(2,3-*f*) quinoline-4,5-dione

2. MATERIALS AND METHODS

2.1. *Production of antiserum*

PQQ was conjugated to gelatin essentially as reported in [9].

The anti-PQQ immune serum was obtained by immunization of adult New Zealand White rabbits with weekly subcutaneous injections of PQQ-gelatin (1 mg PQQ) dissolved in 0.5 ml of 0.1 M PBS, pH 7.2, and 0.5 ml of Freund's complete adjuvant. The rabbits were injected for four consecutive weeks. A booster injection of 2 mg PQQ was delivered 15 days after the last inoculation. Five days later the rabbits were bled from the ear artery for four successive days (25 ml blood/day).

2.2. *Immune dot-blot assay*

Different amounts of PQQ-gelatin were adsorbed on nitrocellulose sheets following the Bio-Rad immunoblot procedure. Specific anti-PQQ antiserum (1/10000) was used as the first antibody. Goat anti-rabbit IgG-horseradish peroxidase conjugate and peroxidase substrates (1-chloro-4-naphthol and hydrogen peroxide) were used to detect the immune complex.

2.3. *Enzyme-linked immunoadsorbent assay*

The procedure adopted was that of Green et al. [10]. Polystyrene 96 wells microtiter plates (3040 F Microtest from Falcon) were coated with varying amounts of PQQ-gelatin containing between 1 and 200 ng PQQ. 50 μl of antiserum (1/5000)

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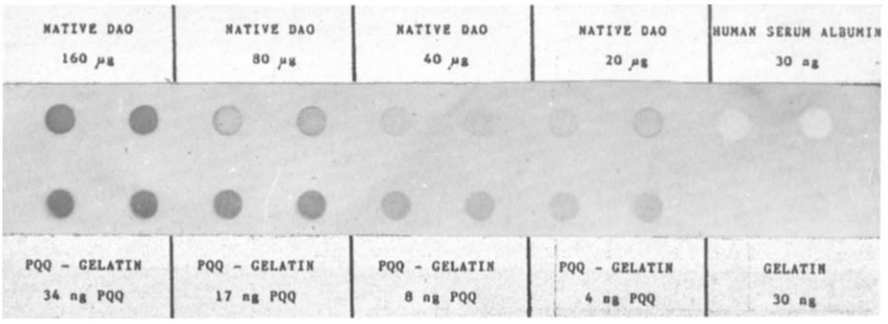

Fig.1. Reaction of LSAO with anti-PQQ antibodies as detected by dot-blot. Increasing concentrations of PQQ-gelatin and LSAO (DAO) as indicated were tested against anti-PQQ antibodies. The color developed in the reaction is proportional to the amount of PQQ present in the test. Gelatin and human serum albumin gave negative results at all concentrations tested.

were added to each well. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad), diluted 1000-fold was used as the second antibody with *o*-phenylenediamine as substrate.

2.4. *Preparation and activity of lentil seedling amine oxidase (LSAO)*

Amine oxidase was prepared from lentil seedlings as described by Floris et al. [11].

The enzyme activity was determined by following the oxygen uptake at 37°C using a YSI oxygen sensitive electrode. The reaction mixture contained LSAO (50 µg) dissolved in 0.1 M K-phosphate buffer, pH 7.2, as such, or after 1 h incubation at room temperature with anti-PQQ antiserum (dilutions used 1:1 to 1:10).

Putrescine in the concentration range 10–100 µM was used as substrate.

3. RESULTS

The antiserum elicited in rabbits by PQQ-gelatin is able to react with both free and protein-bound PQQ.

Fig.1 shows that the wells containing PQQ-gelatin developed a color proportional to the amount of bound PQQ. The wells containing gelatin alone or serum albumin used as controls remained unstained. A positive reaction proportional to the amount used was obtained with LSAO (fig.1). Several metallo-proteins devoid of PQQ did not react in the dot-blot test. Heat denatured LSAO (data not shown) was also recognised by anti-PQQ antibodies.

The dot-blot analysis allowed only a rough estimate of the PQQ content in LSAO. Thus an ELISA test was set up. Fig.2 shows the standard curve obtained by plotting the concentration of gelatin-bound PQQ toward the enzymic activity. This allowed one to determine the concentration of PQQ reacting with anti-PQQ antibodies in native LSAO. Determinations made in triplicate at three protein concentrations on two different batches of LSAO gave a mean value of 0.96 mol PQQ/140000 g LSAO.

The quantitative determination of PQQ in heat-denatured LSAO was prevented by the low solubility of the protein.

The incubation of LSAO with anti-PQQ antiserum at 1:10 to 1:1 dilutions reduced by 30% to 80% the activity of the enzyme toward putrescine. This inhibition was of non-competitive type (fig.3).

Control experiments showed that unspecific rab-

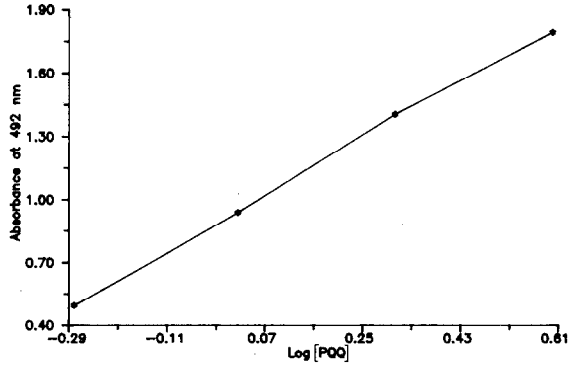

Fig.2. Quantitation of PQQ in LSAO by ELISA. The straight line was obtained by plotting the peroxidase activity versus the log of the amount of PQQ-gelatin used for coating the wells of the microtest plate (5, 27, 54 and 107 ng of PQQ). In this test 5 µg of native LSAO gave an activity corresponding to 12 ng of PQQ. This experiment was repeated three times and the determination was performed in triplicate.

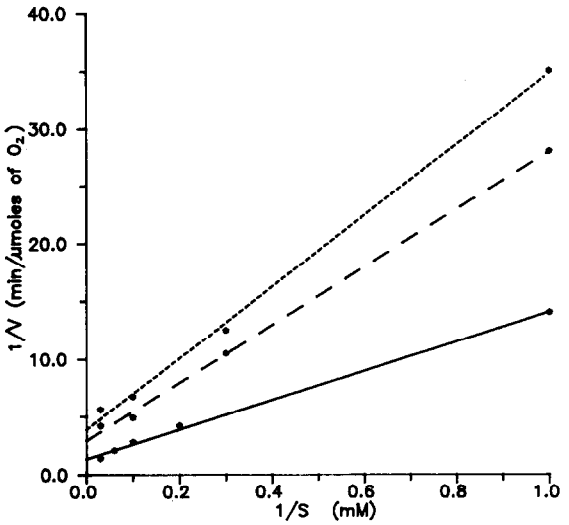

Fig.3. Lineweaver-Burk plot of the LSAO inhibition by anti-PQQ antibodies. LSAO was incubated at room temperature in the absence (continuous line) or presence of the same volume of anti-PQQ antiserum undiluted (dotted line) or diluted 1:2 (dashed line). Then 50 μg of enzyme were diluted in the oxygraph chamber to 3 ml (final volume) with 0.1 M phosphate buffer, pH 7.2, containing the required amount of putrescine. The reaction was started by adding the enzyme and was performed at 37°C. The data were fitted with a least square procedure. The K_m value was invariant in the three cases (0.12 mM).

bit antiserum did not affect the activity of LSAO at any dilution used.

4. DISCUSSION

The presence and role of PQQ in biological systems has only recently been recognized. Nevertheless in the last few years this molecule has gained much attention in view of its diffusion among prokaryotes and eukaryotes [2]. It was found to catalyze such diverse redox reactions as dehydrogenation by methanol dehydrogenase [1], glucose dehydrogenase [12], or choline dehydrogenase [13]; oxidation by copper-containing amine oxidases [14–19]; hydroxylation by dopamine β-hydroxylase [20]; oxygenation by lipoxygenase [21] and even non-oxidative reactions like decarboxylation of DOPA [22] or hydration of nitriles [23]. Despite the impressive diffusion among living organisms, little is known about the metabolism of PQQ. Furthermore the exponential growth of papers reporting its presence in new enzymes and/or different organisms indicates the need for new, fast analytical methods allowing detection and quantitation of PQQ in biological samples.

In this paper we report for the first time the production of a specific antibody allowing detection of PQQ in lentil seedling amine oxidase. LSAO belongs to the class of copper-containing amine oxidases (Cu-AOs) [24], some of which have already been reported to contain PQQ. However, LSAO is the first plant amine oxidase where the presence of PQQ has been determined by a method other than spectroscopic. Furthermore the ELISA method allows the determination of 1 PQQ per dimer of LSAO. This finding is at variance with previous reports concerning the number of active site carbonyl groups in LSAO [25], but is in keeping with the amount of PQQ found in other amine oxidases [2]. It should be pointed out that the reaction of LSAO-bound PQQ with antiserum might be incomplete due to the partial accessibility of the coenzyme to the antibody.

In any case the PQQ covalently bound to LSAO is recognized by the polyclonal antibodies elicited against PQQ covalently bound to gelatin. Since the binding of PQQ to gelatin and to LSAO is not known in detail, the reaction between anti-PQQ antiserum and LSAO does not give structural information on the active site of the enzyme. In this work we have found that the binding of antiserum to LSAO inhibits the enzymic activity in a non-competitive way with respect to the substrate putrescine (fig.3).

The production of monoclonal antibodies might, in the future, allow the discrimination among various types of interaction between PQQ and proteins.

The polyclonal anti-PQQ antibody is, however, a powerful analytical tool with which to investigate the presence and distribution of PQQ in cells and tissues in normal and pathological states like growth, differentiation, neoplastic transformation and fibrosis. Each of these conditions has been indeed reported to involve an altered expression of PQQ containing enzymes [26]. In this line, preliminary immunoblot tests using this polyclonal antibody enabled the detection of the presence of PQQ secreted in the growth medium by *Pseudomonas* sp. [27].

Furthermore, the metabolic studies on PQQ in cell cultures and whole animals may derive very useful support from the use of this antiserum.

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